

AD _____

Award Number: W81XWH-05-1-0134

TITLE: Rapid Identification of Key Pathogens in Wound Infection by Molecular Means

PRINCIPAL INVESTIGATOR: Sydney Finegold, M.D.
Robert Bennion, M.D.

CONTRACTING ORGANIZATION: Brentwood Biomedical Research Institute
Los Angeles, CA 90073

REPORT DATE: January 2006

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

20060606091

REPORT DOCUMENTATION PAGEForm Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. **PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.**

1. REPORT DATE (DD-MM-YYYY) 01-01-2006		2. REPORT TYPE Annual		3. DATES COVERED (From - To) 15 DEC 2004 - 14 DEC 2005	
4. TITLE AND SUBTITLE Rapid Identification of Key Pathogens in Wound Infection by Molecular Means				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-05-1-0134	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Sydney Finegold, M.D. Robert Bennion, M.D. E-mail: sidfinegol@aol.com				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Brentwood Biomedical Research Institute Los Angeles, CA 90073				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT The purpose of this study is to develop a real-time PCR-based high throughput detection and identification method for important wound pathogens. This will enable us to provide accurate identification a great deal sooner than has been possible in the past. This is likely to result in improved clinical outcome, less chance of bacterial resistance, and cost savings. To date, we have developed over 20 group- or species-specific primer/probe sets for pathogens encountered in wound infection. The specificities of the primer/probe sets were successfully evaluated with a battery of bacteria. The evaluation of sensitivity is underway. When the entire set of primers and probes has been completed, we will be able to detect, identify and semi-quantitate the important wound pathogens within 8 hours using this TaqMan PCR assay.					
15. SUBJECT TERMS Wound infection, bacteria, molecular identification, real-time PCR					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	18. NUMBER OF PAGES 21	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER (include area code)

Table of Contents

Cover.....	1
SF 298.....	2
Introduction.....	4
Body.....	4-18
Key Research Accomplishments.....	18
Reportable Outcomes.....	18
Conclusions.....	18
References.....	19-21

INTRODUCTION

Wound infection remains an important problem in both military and civilian life despite availability of a wide array of antimicrobial agents and improvements in surgical technique. Wound infection leads to morbidity and, at times, mortality. Studies have shown that infection of post-surgical wounds adds 10 days to the period of hospitalization and approximately \$4,000 to the cost [1,2]. Severe trauma and foreign bodies add to the difficulties in management of military wounds and some civilian wounds. Other problems in wound management relate to the increasing numbers of immunosuppressed patients, the use of extensive surgical procedures in certain situations, and the increasing incidence of microorganisms resistant to antimicrobial agents.

Most wound infections involve both aerobic or facultative bacteria and anaerobic or microaerophilic bacteria. The latter category of organisms often have fastidious growth requirements and may therefore be readily overlooked. The difficulties and time required for good anaerobic bacteriology, along with decreases in available funding, have led many laboratories to do abbreviated workups for anaerobes or to eliminate anaerobic bacteriology entirely. As few as 50 to 75% of anaerobes from various sources are characterized satisfactorily, according to a recent survey [3] and 27% of hospital microbiology laboratories indicated that they never identified anaerobes. Furthermore, virtually all laboratories depend on phenotypic testing alone for identification, typically using commercial kits. These conventional identification protocols are not only laborious and time-consuming, but also often result in inconclusive or even inaccurate identification. In addition, they are culture-dependent and thus more time-consuming. Anaerobic bacteria grow more slowly, but more importantly it takes considerable time to get the various elements of a mixed infection (commonly involving 4 to 12 bacteria) isolated in pure culture so that identification and antimicrobial susceptibility testing can proceed. These delays and limitations can lead to inappropriate antibiotic use in wound infection patients or use of unnecessarily broad-spectrum agents, which contributes to increasing rates of antibiotic resistance. If empirical treatment is ineffective, the patient may deteriorate. The development and application of molecular diagnostic techniques has initiated a revolution in the diagnosis and monitoring of infectious diseases [4]. Rapid diagnosis can be achieved by the direct detection of characteristic bacterial genes in clinical specimens. In recent years, real-time PCR has been applied successfully in the medical field, for example in the quantitation of various DNA and RNA viruses in patients [5-9], in the detection of gene amplification [10], gene mutations [11,12], or chromosomal rearrangements, in the quantitation of gene expression [10,12], and in quantitation of particular pathogens in clinical specimens [13-19]. Here, we describe the use of real-time PCR (TaqMan) assay for high throughput detection of wound infection pathogens.

BODY

[1]: Key pathogens involved in wound infections:

Anaerobes

Gram-positive cocci

Peptostreptococcus micros, *P. asaccharolyticus*, *P. anaerobius*, *Finegoldia magna*

Gram-negative rods:

Prevotella spp., *P. melaninogenicus*, *P. nigrescens*, *P. bivia*, *P. disiens*

Fusobacterium spp., *F. nucleatum*, *F. mortiferum*

Bacteroides fragilis group: *Bacteroides fragilis*, *B. thetaiotaomicron*, *B. vulgatus*, *B. ovatus*, *B. uniformis*, *B. distasonis*

Bacteroides gracilis

Bilophila wadsworthia

Porphyromonas macacae

Gram-positive rods:

Actinomyces odontolyticus, *A. viscosus*, *A. naeslundii*, *A. israelii*

Clostridium perfringens, *C. ramosum*, *C. sordellii*, *C. novyi*

C. clostridioforme group: *C. bolteae*, *C. clostridioforme*, and *Clostridium hathewayi*

Aerobes

Gram-positive cocci:

Streptococci:

Group A streptococci (*Streptococcus pyogenes*)

Group B streptococci (*Streptococcus agalactiae*)

Streptococcus anginosus group: *S. anginosus*, *S. constellatus* subsp. *constellatus*, *S. intermedius*

Staphylococci:

Staphylococcus spp., *S. aureus*, *S. epidermidis*

MRSA (Methicillin Resistant *Staphylococcus aureus*)

MSSA (Methicillin Sensitive *Staphylococcus aureus*)

PVL toxin (Panton Valentine Leukocidin toxin)

Enterococci:

Enterococcus faecium, *E. faecalis*

Gram-negative rods:

Enterobacter cloacae, *E. aerogenes*

Escherichia coli

Klebsiella oxytoca, *K. pneumoniae*

Pseudomonas aeruginosa

[2] TaqMan probe technology:

The Taqman probe technology combines the features of rapid PCR and real-time detection of an amplification product. It uses the 5'-nuclease assay and FRET (fluorescent resonance energy transfer). The hydrolysis probe is conjugated with a quencher fluorochrome, which absorbs the fluorescence of the reporter fluorochrome as long as the probe is intact. However, upon amplification of the target sequence, the hydrolysis probe is displaced and subsequently hydrolyzed by the *Taq* polymerase. This results in the separation of the reporter and quencher fluorochrome and consequently the fluorescence of the reporter fluorochrome becomes detectable. During each consecutive PCR cycle this fluorescence will further increase because of the progressive and exponential accumulation of free reporter fluorochromes.

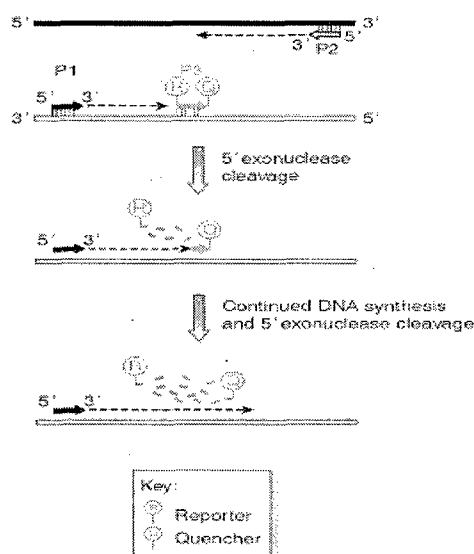


Fig.1. In addition to two conventional PCR primers, P1 and P2, which are specific for the target sequence, a third primer, P3, is designed to bind specifically to a site on the target sequence downstream of the P1 binding site. P3 is labelled with two fluorophores, a reporter dye (R) is attached at the 5' end, and a quencher dye (D), which has a different emission wavelength to the reporter dye, is attached at its 3' end. Because its 3' end is blocked, primer P3 cannot by itself prime any new DNA synthesis. During the PCR reaction, *Taq* DNA polymerase synthesizes a new DNA strand primed by P1 and as the enzyme approaches P3, its 5'–3' exonuclease activity progressively degrades the P3 primer from its 5' end. The end result is that the nascent DNA strand extends beyond the P3 binding site and the reporter and quencher dyes are no longer bound to the same molecule. As the reporter dye is no longer in close proximity to the quencher, the resulting increase in reporter emission intensity is easily detected

Real-time PCR has several advantages over conventional PCR. It focuses on the logarithmic phase of product accumulation rather than on the end-product abundance. Therefore, it is more accurate since it is less affected by amplification efficiency or depletion of a reagent. In addition, it has an increased dynamic range for quantification of target sequence (at least 5 orders of magnitude). Furthermore, without any post-PCR manipulation of the samples, cross-contamination between samples is greatly reduced. Finally, PCR results can be obtained within 2 hours.

(1). ABI PRISM® 7500 Sequence Detection System

ABI PRISM® 7500 Sequence Detection System (Applied Biosystems, Foster City, Calif.) was purchased, set up and standardized. This system integrates a PCR-based assay with laser scanning technology to excite fluorescent dyes present in the specially designed TaqMan® probes. It is a fully integrated system for real-time detection of PCR. The system includes a built-in thermal cycler, a laser to induce fluorescence, CCD (charge-coupled device) detector, real-time sequence detection software, and TaqMan® reagents for the fluorogenic 5' nuclease assay. All the personnel involved in this project were trained.

(2). TaqMan Primer and Probe design:

Generally, putative genus- or species-specific primer and probe sets were chosen by examining the alignment of the target sequences with sequences of similar organisms

and selecting sequences unique to the organism(s) in question. Sequence alignment was done by using CLUSTAL-W [20] (<http://genome.kribb.re.kr>). Regions that are unique to sequences representing different genera or species were selected for primer and probe design. All primer and probe sets were subjected to the guidelines established by Applied Biosystems by using Primer Express 2.0 (Applied Biosystems, Foster City, Calif.) The primer and probe sequences were analyzed for T_m (melting temperature), secondary structure formation, G+C content, and primer-dimer formation with the NetPrimer analysis software <http://www.premierbiosoft.com/netprimer>. We compared potential candidates for PCR primers and probes to the aligned SSU_rRNA database of the Ribosomal Database Project using the CHECK_PROBE utility [21] and to all available sequences by using the BLAST database search program (www.ncbi.nlm.nih.gov/BLAST) [22]. All the primers and probes designed to date are summarized in **Table 1**.

16S rRNA gene:

The genus- or species-specific primer and probe sets finished to date in this study were designed from the 16S rRNA gene for all the following bacteria:

Bacteroides fragilis group species: *B. fragilis*, *B. stercoris*, *B. vulgatus* and *B. thetaiotaomicron*
Bilophila wadsworthia
Prevotella species including *P. disiens*, *P. bivia*, *P. melaninogenica*, and *P. nigrescens*
Porphyromonas macacae (& *Porphyromonas salivosa*)
 Gram-positive anaerobic cocci (GPAC) including *Peptostreptococcus anaerobius*, *P. micros* and *Finegoldia magna*
Actinomyces odontolyticus
Pseudomonas aeruginosa

Spy 1258 gene:

A *S. pyogenes*-specific gene (*Spy* 1258) that encodes a putative transcriptional regulator [23] was used for *Streptococcus pyogenes* (Group A streptococcus) specific-primer and probe design. It was noted that a stretch of nucleotides identical to *Spy* 1258 (nt. 6651-7193) was also found in the complete genome of *S. pyogenes* M3 strains MGAS315 (GenBank accession No. AE014154) and SSI-1 (GenBank accession No. AP005144) as well as M18 strain MGAS8232 (GenBank accession No. AE010045). However, the *Spy* 1258 gene sequence was clearly absent in other bacterial genomes available at GenBank. The primer and probe set was subjected to the guidelines established by Applied Biosystems (Foster City, Calif.) by using Primer Express software (V.2.0).

sip gene:

The primers and probes specific for group B streptococcus (GBS)-*Streptococcus agalactiae* were designed using the *sip* gene [24] which encodes Sip-surface immunogenic protein as this protein is universally expressed in GBS. *S. agalactiae*-

specific primers and probe were designed using Primer Express 2.0 from Applied Biosystems.

pbp2b gene:

The partial penicillin-binding protein 2B gene (*pbp2b* gene) [25] was used for the anginosus group streptococci (AGS)(formerly called "*Streptococcus milleri*")-specific primer and probe design. The primers and probes were designed according to the alignment analysis of *pbp2b* gene sequences of *Streptococcus anginosus* strain MAS624 (GenBank Accession No. AY289802), *Streptococcus intermedius* strain ATCC 27335 (AY289801), *Streptococcus constellatus* subsp. *pharyngis* strain MM9889a (AY289800), *Streptococcus anginosus* strain ATCC 33397 (AY289798), *Streptococcus constellatus* subsp. *constellatus* strain ATCC 27823 (AY289799) with the homologous genes of *Streptococcus mitis* (Z22182), *Streptococcus pneumoniae* (Z22184), *Streptococcus mutans* (AE014903) and *Streptococcus agalactiae* (AL766847).

tuf gene:

The *tuf* gene encodes the elongation factor Tu, which is an essential constituent of the bacterial genome [26]. The *tuf* gene sequences of *Staphylococcus* species (*S. aureus* [AF298796], *S. epidermidis* [AF298800], *S. saprophyticus* [AF298804], *S. haemolyticus* [AF298801], *S. hominis* [AF298802], *S. lugdunensis* [AF298803], *S. simulans* [AF208805], *S. warneri* [AF298806], *S. xylosus* [AY763438], *S. sciuri* [AY763434], *S. cohnii* subsp. *ureolyticus* [AY298799] and *S. capitis* [AF298798]) that were available from GenBank were analyzed by the software mentioned above.

nuc gene:

S. aureus strains produce an extracellular thermostable nuclease (TNase) with a frequency similar to that at which they produce coagulase. Tnase protein has been well characterized and its gene, the *nuc* gene, has been cloned and sequenced [27]. The *nuc* gene is unique to *S. aureus* and it was used for primer and probe design.

(3). TaqMan real-time PCR:

Real-time PCR was performed on the ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, Calif.). After primers and probes were designed, they were synthesized by Sigma-genosys. Probes were labeled with the reporter dye 6-carboxyfluorescein (6'-FAM) at the 5' end and with the quencher dye 6-carboxytetramethylrhodamine (TAMRA) at the 3' end. TaqMan real-time PCR was performed with 3 µl of DNA, 12.5 µl of 2X TaqMan PCR master mix (Applied Biosystems, Foster City, Calif.), 900 nmol of each primer, and a 200-nmol probe in a final volume of 25 µl. Thermal cycling conditions were as follows: 2 min at 50°C, 10min at 95°C followed by 45 repeats of 15 s at 95°C, and 1min at 60°C. Data collection was performed during each annealing phase.

Table 1. Primers and probes included in this study

Target organism	Sequence of primer or probe genes (5' end labeled with FAM-6 and 3' end labeled with TRAMA)	Target	Tm	GC%	Length	Reference
<i>*B. fragilis</i>	Bfrag-F: 5'-GCGGGTGACCGTATGCTAAT-3'	16s	60.0	55.0	20	This study
	Bfrag-R: 5'-TCACGAAGTCGGGTGCA-3'	rRNA	58.4	55.5	18	
	Bfrag-P: 5'-CCAAAATCCTCTCTCAGTTCGGATCGAAG-3'		71.5	48.2	29	
<i>*B. thetaiotaomicron</i>	Bthe-F: 5'-CGGGCTTAAATTGCATTG-3'	16s	57.1	42.1	19	This study
	Bthe-R: 5'-CCATGCAGCACCTTCACATT-3'	rRNA	59.0	50.0	20	
	Bthe-P: 5'-ATAATCTGGAAACAGGTAGCCGCAAGG-3'		70.0	46.4	28	
<i>*B. stercoris</i>	Bster-F: 5'-TTGCAACTGACTGAATCGGAA-3'	16s	59.0	42.8	21	This study
	Bster-R: 5'-CTGACGACAACCATGCAGCA-3'	rRNA	60.9	55.0	20	
	Bster-P: 5'-CGGTTCTTCTTCCTTCGGACAGTTGTGAA-3'		70.6	48.1	27	
<i>*B. vulgatus</i>	Bvul-F: 5'-CGGGCTTAAATTGCAGATGA-3'	16s	58.9	45.0	20	This study
	Bvul-R: 5'-CATGCAGCACCTTCACAGAT-3'	rRNA	57.4	50.0	20	
	Bvul-P: 5'-GGTGAAAGCCGTAAAGCCGCAAGG-3'		70.8	60.8	23	
<i>*Bilophila wadsworthia</i>	Bwads-F: 5'-GGCTGGAAACGGTCGCTAA-3'	16s	61.0	57.8	19	This study
	Bwads-R: 5'-GGACTCATCTCTTAAGCGATAGC-3'	rRNA	57.9	50.0	22	
	Bwads-P: 5'-CGAATACGCTCCCGATTTTATCATTTGGG-3'		72.2	46.4	28	
<i>Prevotella</i> spp.	Prev-F: 5'-CCAGCCCAAGTAGCGTGCA-3'	16s	58.6	61.1	18	[28]
	Prev-R: 5'-TGGACCTTCCGTATTACCGC-3'	rRNA	60.0	55.0	20	
	Prev-P: 5'-CAAATCTGATGCCGTCAATCGAAGACTATGC-3'		72.7	46.7	30	
<i>*P. nigrescens</i>	Pnig-F: 5'-GGCCTAATACCCGATGTGTTT-3'	16s	58.5	47.6	21	This study
	Pnig-R: 5'-ACGCATCCCCATCCCTTA-3'	rRNA	58.1	55.5	18	
	Pnig-P: 5'TGACGGCATCCGATATGAAACAAAGG-3'		70.0	46.1	26	
<i>Pseudomonas aeruginosa</i>	Pseu-F: 5'- TGCGATCACCACTTCTACTT-3'	16s	58.9	47.6	21	This study
	Pseu-R: 5'- TTTCGCGTGTACGTCCAGA-3'	rRNA	59.0	52.6	19	
	Pseu-P: 5'-AGTACGACAGCTCCGACCTGAAGCCG-3'		71.2	61.5	26	

Target organism	Sequence of primer or probe genes (5' end labeled with FAM-6 and 3' end labeled with TRAMA)	Target	Tm	GC%	Length	Reference
<i>*P. distiens</i>	Pdis-F: 5'-GCCGGTACAGAAATGTTGGTT-3'	16S	57.4	50.0	20	present
	Pdis-R: 5'-GGTTCGTGAGGTCGGGT-3'	rRNA	57.6	61.1	18	study
<i>*P. bivia</i>	Pdis-P: 5'-AATGTAATCTAATCTTAAAGCCGGTCCCAGTTC-3'		71.5	38.2	34	
	Pbiv-F: 5'-GGTGGTACAGATAGTTGGTCGTG-3'	16S	58.8	52.1	23	present
	Pbiv-R: 5'-TTGCAGACCCCAAGTCCGA-3'	rRNA	59.9	61.1	18	study
<i>P. melaninogenica</i>	Pbiv-P: 5'-GCAAAATACGATCTAATCCTTAAACCAATCTCTCAG-3'		69.5	35.3	34	
	Pmel-F: 5'-GTGGGATAACCTGCCGAAAG-3'	16S	59.6	55.0	20	[28]
	Pmel-R: 5'-CCCATCCATTACCGATAAATCTTTA-3'	rRNA	61.2	36.0	25	
<i>*Porphyromonas macacae</i>	Pmel-P: 5'-GCATAGTCTTCGATGACGGCATCAGATTG-3'		72.7	46.6	30	
	Pmac-F: 5'-TTGAAATTTAGCGGACTATGTATG-3'	16S	56.8	33.3	24	present
	Pmac-R: 5'-AGCTGACGACAACCATGCA-3'	rRNA	57.6	52.6	19	study
	Pmac-P: 5'-TACATATCCTGTCTCACAAAGCCGCTAAGTAGG-3'		70.9	48.3	31	
<i>Fusobacterium</i> spp.	Fuso-F: 5'-AAGCGCGTCTAGGTGTTATGT-3'	16S	65.8	50.0	22	[28]
	Fuso-R: 5'-TGATGTTCCGCTTACCTCTCCAG-3'	rRNA	60.6	52.1	23	
<i>*Finegoldia magna</i>	Fuso-P: 5'-CAACGCAATACAGAGTTGAGCCCTGCATT-3'		73.1	48.2	29	
	Fmag-F: 5'-AGGGTACGCAAGCGGTTTAAAT-3'	16S	62.8	52.3	21	present
	Fmag-R: 5'-TCAGTTTCCAAATGCTTTACGG-3'	rRNA	58.2	42.8	21	study
<i>*Micromonas micros</i>	Fmag-P: 5'-AGTCGAATGTAAAGATCGGGGCTCAAC-3'		70.0	46.4	28	
	Pmic-F: 5'-GCCGTTGAAACGACGATT-3'	16S	61.0	50.0	20	present
	Pmic-R: 5'-CACCGATAAATCTTTGACCCCTAT-3'	rRNA	60.9	41.6	24	study
<i>*P. anaerobius</i>	Pmic-P: 5'-TACCGCATGAGACCCACAGAAATCGCA-3'		70.4	52.0	25	
	Pana-F: 5'-GGCGTAAAGGGTGCCTAGGT-3'	16S	61.7	60.0	20	present
	Pana-R: 5'-TTCGGAGGCTAACTACGGTTGA-3'	rRNA	61.6	50.0	24	study
<i>*Actinomyces odontolyticus (& A. lingnae)</i>	Pana-P: 5'-TCTTTCAAGTCGGTGGTTAAAGGCTACGG-3'		71.6	48.2	29	
	Aodo-F: 5'-ATGTGGTGGCATTTAGTTGGTC-3'	16S	59.0	45.4	22	present
	Aodo-R: 5'-GACTTAAACCAACATCTCACGAC-3'	rRNA	58.1	47.8	23	study
	Aodo-P: 5'-GGTGGTGCATGGTTGTCGTCAGCTCG-3'		75.4	61.5	26	

<i>Clostridium perfringens</i>	CPerF165F: 5'-CGCATAAACGTTGAAAGATGG-3'	16S	56.8	45.0	20	[29]
	CPerF269R: 5'-CCTTGGTAGGCCGTTACCC-3'	rRNA	59.4	63.1	19	
	CperF-P: 5'-TCATCATTC AACCCAAAGGAGCAAATCC-3'		67.5	42.3	26	
* <i>Staphylococcus</i> spp.	Staph-F: 5'-CAAC(T/A)CCAGAACGTGAT(T/C)CTG-3'	tuf	58.6	52.6	21	present study
	Staph-R: 5'-CAGTACCAACGACCAAGTATTGAG-3'	gene	59.6	52.1	23	
	Staph-P: 5'-AAACCAATTCATGATGCCAGTTGAGGACG-3'		72.0	46.4	28	
* <i>S. aureus</i>	Saur-F: 5'-AGCTCAGCAAATGCATCACA-3'	nuc	59.2	42.8	21	present study
	Saur-R: 5'-TTAGTTGAAGTTGCACTATATACTGTTGGAT-3'	gene	62.6	32.2	31	
	Saur-P: 5'-CAGATAA(C/T)GGCGTAAATAGAAAGTGTTTC-3'		67.5	40.7	32	
<i>S. epidermidis</i>	Sepi-F: 5'-TACACACCGCCCGTCACA-3'	16S	59.0	61.0	18	[30]
	Sepi-R: 5'-CTTCGACGGGCTAGCTCCAAAT-3'	rRNA	64.7	54.5	22	
	Sepi-P: 5'-CACCCGAAGCCGGTGGAGTAACC-3'		70.5	65.2	23	
MRSA	MecA-F: 5'-GGCAAATATTACCGCACCTCA-3'	MecA	58.6	50.0	20	[31]
	MecA-R: 5'-GTCTGCCACTTCTCCTTGT-3'	gene	53.9	50.0	20	
	MecA-P: 5'-AFATCTTATGCAAACTTAATTGGCAAATCC-3'		69.0	32.0	30	
PVL	PVL-F: 5'-ACACACTATGGCAATAGTTATT-3'	PVL	51.4	31.4	23	[31]
	PVL-R: 5'-AAAGCAATGCAATTGATGTA-3'	gene	51.8	30.0	20	
	PVL-P: 5'-ATTTGTAAACAGAAAATTACACAGTTAAATATGA-3'		61.67	21.0	33	
*Group A <i>Streptococcus</i>	Spyo-F: 5'-CAAAAATGACACTCTGGATGATTG-3'	Spy	60.7	36.0	25	present study
	Spyo-R: 5'-GGACAAAGTTTGTATTGAGGACTTG-3'	1258	61.9	45.8	24	
	Spyo-P: 5'-CCGTTTGTAAATCAGGCTGAAATCTACACAGAC-3'		72.1	41.1	34	
*Group B <i>Streptococcus</i>	GBS-F: 5'-TCAGTCGCAAGTGTTC AAGCA-3'	Sip	60.0	47.6	21	present study
	GBS-F: 5'-AAATCAGCCTTTACCTCTGAAACAG-3'	gene	60.0	40.0	25	
	GBS-P: 5'-AAGAAACAGATACGACGTGGACAGCACGT-3'		71.2	48.2	29	
*Anginosus group streptococci	SAG-F: 5'-CGGTAGCTAATGG(T/C)GGTACTCGTA-3'		64.0	50.0	28	present study
	SAG-R: 5'-ATTTTCCAAG(A/C)CC(A/G)CC(T/C)(T/G)T(T/C)TCAT-3'	Pbp2b	64.4	33.3	24	
	SAG-P: 5'-GCACC(A/G)CACTTAGT(G/A)GAAAGGAATTTATGACAA-3'	gene	74.9	42.4	33	

* Primers and probes were designed in present study.

(4). Specificity evaluation for TaqMan PCR assay:

The specificities of primer/probe sets designed in this study were predicted by comparison to the aligned SSU_rRNA database of the RDP using the CHECK_PROBE utility [21] and to all available sequences by using the BLAST database search program (www.ncbi.nlm.nih.gov/BLAST) [22], and further confirmed by testing against a panel of phylogenetically related bacterial strains. The specificities of the rest of the primer/probe sets have previously been demonstrated [28-32].

Gram-negative anaerobes:

The specificities of the primer and probe sets for *Bacteroides fragilis*, *B. thetaiotaomicron*, *B. stercoris*, *B. vulgatus*, *Bilophila wadsworthia*, *Prevotella nigrescens*, *P. disiens*, *P. bivia* and *Porphyromonas macacae* were tested against a panel of genomic DNA preparations (Table 2 & 3) from type strains and well-characterized clinical isolates that are phylogenetically related to these species. The primer/probe sets detected only corresponding strain(s), except for that of *B. ovatus* which showed a significant increase in fluorescence indicating cross-reactivity with *B. thetaiotaomicron* species-specific primer and probe. No signal was detected for any other non-corresponding strains.

Table 2. Bacterial strains tested by TaqMan assay

Species	Strains	No. of Strains	Bthe-F/R/P	Bfrag-F/R/P	Bster-F/R/P	Bvul-F/R/P	Pnig-F/R/P	Pdis-F/R/P	Pbiv-F/R/P
<i>Bacteroides thetaiotaomicron</i>	ATCC 29148 ^T	1	+	-	-	-	NT	NT	NT
<i>Bacteroides thetaiotaomicron</i>	Clinical isolates	6	+	-	-	-	NT	NT	NT
<i>Bacteroides ovatus</i>	ATCC 8483 ^T	1	+	-	-	-	NT	NT	NT
<i>Bacteroides ovatus</i>	Clinical isolates	6	+	-	-	-	NT	NT	NT
<i>Bacteroides fragilis</i>	ATCC 25285 ^T	1	-	+	-	-	NT	NT	NT
<i>Bacteroides fragilis</i>	Clinical isolates	6	-	+	-	-	NT	NT	NT
<i>Bacteroides stercoris</i>	ATCC 43183 ^T	1	-	-	+	-	NT	NT	NT
<i>Bacteroides stercoris</i>	Clinical isolates	6	-	-	+	-	NT	NT	NT
<i>Bacteroides vulgatus</i>	ATCC 8482 ^T	1	-	-	-	+	NT	NT	NT
<i>Bacteroides vulgatus</i>	Clinical isolates	6	-	-	-	+	NT	NT	NT
<i>Bacteroides distasonis</i>	ATCC 8503 ^T	1	-	-	-	-	NT	NT	NT
<i>Bacteroides merdae</i>	ATCC 43184 ^T	1	-	-	-	-	NT	NT	NT
<i>Bacteroides caccae</i>	ATCC 43185 ^T	1	-	-	-	-	NT	NT	NT
<i>Bacteroides uniformis</i>	ATCC 8492 ^T	1	-	-	-	-	NT	NT	NT
<i>Bacteroides eggerthii</i>	ATCC 27754 ^T	1	-	-	-	-	NT	NT	NT
<i>Bacteroides goldsteinii</i>	ATCC BAA-1180 ^T	1	-	-	-	-	NT	NT	NT

Table 2 (Cont.)

<i>Bacteroides nordii</i>	ATCC BAA- 998 ^T	1	-	-	-	-	NT	NT	NT
<i>Bacteroides salyersiae</i>	ATCC BAA-997 ^T	1	-	-	-	-	NT	NT	NT
<i>Prevotella nigrescens</i>	ATCC 33563 ^T	1	NT	NT	NT	NT	+		-
<i>Prevotella nigrescens</i>	Clinical isolates	5	NT	NT	NT	NT	+		-
<i>Prevotella disiens</i>	ATCC 29426 ^T	1	NT	NT	NT	NT	-	+	-
<i>Prevotella disiens</i>	Clinical isolates	5	NT	NT	NT	NT	-	+	-
<i>Prevotella bivia</i>	ATCC 29303 ^T	1	NT	NT	NT	NT	-	-	+
<i>Prevotella bivia</i>	Clinical isolates	3	NT	NT	NT	NT	-	-	+
<i>Prevotella melaninogenica</i>	ATCC 25845 ^T	1	NT	NT	NT	NT	-	-	-
<i>Prevotella buccae</i>	ATCC 33574 ^T	1	NT	NT	NT	NT	-	-	-
<i>Prevotella buccalis</i>	ATCC 35310 ^T	1	NT	NT	NT	NT	-	-	-
<i>Prevotella corporis</i>	ATCC 33547 ^T	1	NT	NT	NT	NT	-	-	-
<i>Prevotella denticola</i>	ATCC 33185	1	NT	NT	NT	NT	-	-	-
<i>Prevotella intermedia</i>	ATCC 25611 ^T	1	NT	NT	NT	NT	-	-	-
<i>Prevotella loescheii</i>	ATCC 15930 ^T	1	NT	NT	NT	NT	-	-	-
<i>Prevotella oralis</i>	ATCC 33269 ^T	1	NT	NT	NT	NT	-	-	-
<i>Prevotella oulorum</i>	ATCC 43324 ^T	1	NT	NT	NT	NT	-	-	-
<i>Prevotella veroralis</i>	ATCC 33779 ^T	1	NT	NT	NT	NT	-	-	-
<i>Prevotella zooglyphiformans</i>	ATCC 33285 ^T	1	NT	NT	NT	NT	-	-	-
<i>Porphyromonas gingivalis</i>	ATCC 33277 ^T	1	NT	NT	NT	NT	-	-	-
<i>Porphyromonas endodontalis</i>	ATCC 35406 ^T	1	NT	NT	NT	NT	-	-	-

Table 3. Bacterial strains tested by TaqMan assay

Species	Strains	No. of Strains	Pmac-F/R/P	Bwads-F/R/P
<i>Porphyromonas macacae</i>	ATCC 33141 ^T	1	+	NT
<i>Porphyromonas asaccharolytica</i>	ATCC 25260 ^T	1	-	NT
<i>Porphyromonas endodontalis</i>	ATCC 35406 ^T	1	-	NT
<i>Porphyromonas levii</i>	ATCC 29147 ^T	1	-	NT
<i>Porphyromonas gingivalis</i>	ATCC 33277 ^T	1	-	NT

<i>Porphyromonas gingivalis</i>	ATCC 49417	1	-	NT
<i>Porphyromonas cansulci</i>	NCTC 12858 ^T	1	-	NT
<i>Porphyromonas cangingivalis</i>	NCTC 12856 ^T	1	-	NT
<i>Porphyromonas canoris</i>	NCTC 12835 ^T	1	-	NT
<i>Porphyromonas somerae</i>	ATCC BAA-1230 ^T	1	-	NT
<i>Porphyromonas uenonis</i>	ATCC BAA-906 ^T	1	-	NT
<i>Porphyromonas gulae</i>	ATCC 51700 ^T	1	-	NT
<i>Tannerella forsythensis</i>	ATCC 43037	1	NT	-
<i>Bilophila wadsworthia</i>	ATCC 49260 ^T	1	NT	+
<i>Bilophila wadsworthia</i>	ATCC 51581	1	NT	+
<i>Bilophila wadsworthia</i>	Clinical isolates	8	NT	+
<i>Desulfovibrio piger</i>	ATCC 29098 ^T	1	NT	-
<i>Desulfovibrio piger</i>	Clinical isolates	4	NT	-
<i>Desulfovibrio desulfuricans</i>	ATCC 7757	1	NT	-
<i>Desulfovibrio desulfuricans</i>	Clinical isolates	4	NT	-
<i>Sutterella wadsworthensis</i>	ATCC 51579 ^T	1	NT	-
<i>Desulfovibrio fairfieldensis</i>	Clinical isolates	2	NT	-
<i>Desulfovibrio vulgaris</i>	Clinical isolates	2	NT	-

NT: not tested

Gram-positive anaerobes:

The specificities of the primer and probe sets for *F. magna*, *M. micros* and *P. anaerobius* were tested by running PCR with DNA samples from 14 type strains and 17 well-characterized clinical isolates that are phylogenetically related to GPAC species; this resulted in amplification only with the DNA from the corresponding GPAC reference strain(s). None of the non-corresponding organisms tested by the PCR showed an increase in fluorescence to indicate cross-activity with the species-specific primer/probe sets (See table 4).

Comparison of the sequences of primer/probe set for *Actinomyces odontolyticus* to the aligned SSU_rRNA database of the RDP using the CHECK_PROBE utility predicted it cross-reacts with *A. lingnae*. Further tests will be done with this set of oligonucleotides in the future.

Table 4: Bacterial strains tested by TaqMan assay

Species	Strains	No.	Fmag-F/R/P	Pmic-F/R/P	Pana-F/R/P
<i>Finegoldia magna</i>	CCUG 17636 ^T	1	+	-	-
<i>Finegoldia magna</i>	Clinical isolates	6	+	-	-
<i>Micromonas micros</i>	ATCC 33270 ^T	1	-	+	-
<i>Micromonas micros</i>	Clinical isolates	6	-	+	-
<i>Peptostreptococcus anaerobius</i>	CCUG 7835 ^T	1	-	-	+
<i>Peptostreptococcus anaerobius</i>	Clinical isolates	5	-	-	+
<i>Anaerococcus hydrogenalis</i>	ATCC 49630 ^T	1	-	-	-
<i>Anaerococcus lactolyticus</i>	CCUG 31351 ^T	1	-	-	-
<i>Anaerococcus octavius</i>	CCUG 38493 ^T	1	-	-	-
<i>Anaerococcus prevotii</i>	CCUG 41932 ^T	1	-	-	-
<i>Anaerococcus tetradius</i>	CCUG 46590 ^T	1	-	-	-
<i>Anaerococcus vaginalis</i>	CCUG 31349 ^T	1	-	-	-
<i>Peptoniphilus asaccharolyticus</i>	CCUG 9988 ^T	1	-	-	-
<i>Peptoniphilus harei</i>	CCUG 38491 ^T	1	-	-	-
<i>Peptoniphilus indolicus</i>	CCUG 17639 ^T	1	-	-	-
<i>Peptoniphilus ivorii</i>	CCUG 38492 ^T	1	-	-	-
<i>Peptoniphilus lacrimalis</i>	CCUG 31350 ^T	1	-	-	-

Gram-positive aerobes:

Anginosus group and Group A and B streptococci:

As the non-anginosus group streptococci, the 14 reference strains representing 12 different species (Table 5) were used. As the anginosus streptococci, the type strain (CCUG 27298^T) and three isolates of *S. anginosus*, the type strain (ATCC 27283^T), one reference strain (CCUG 24889) and one isolate of *S. constellatus subsp. constellatus*, and the type strain (CCUG 32759^T) and two isolates of *S. intermedius* were used. The clinical strains of the anginosus group were recovered from our facility. The clinical isolates were identified by routine biochemical methods, which included evaluation by use of the Vitek and API systems. None of the 14 non-anginosus organisms tested by the PCR showed an increase in fluorescence to indicate cross-reactivity with the primer/probe sets (Table 5). Conversely, all the anginosus streptococci isolates showed an exponential increase in fluorescence. Similarly, the primer/probe sets for Group A and Group B streptococci were tested against a battery of streptococci (Table 5), and fluorescent signal were only detected from corresponding strains.

Staphylococcus spp. & *S. aureus*:

An analysis of the bacterial battery demonstrated the specificities of our primers and probes. The broad-range primer/probe set designed for the *Staphylococcus* genus generated positive curves for all of the staphylococci tested (Fig.2.). The *S. aureus*-specific assay detected only the *S. aureus* isolates in the battery (Table 5).

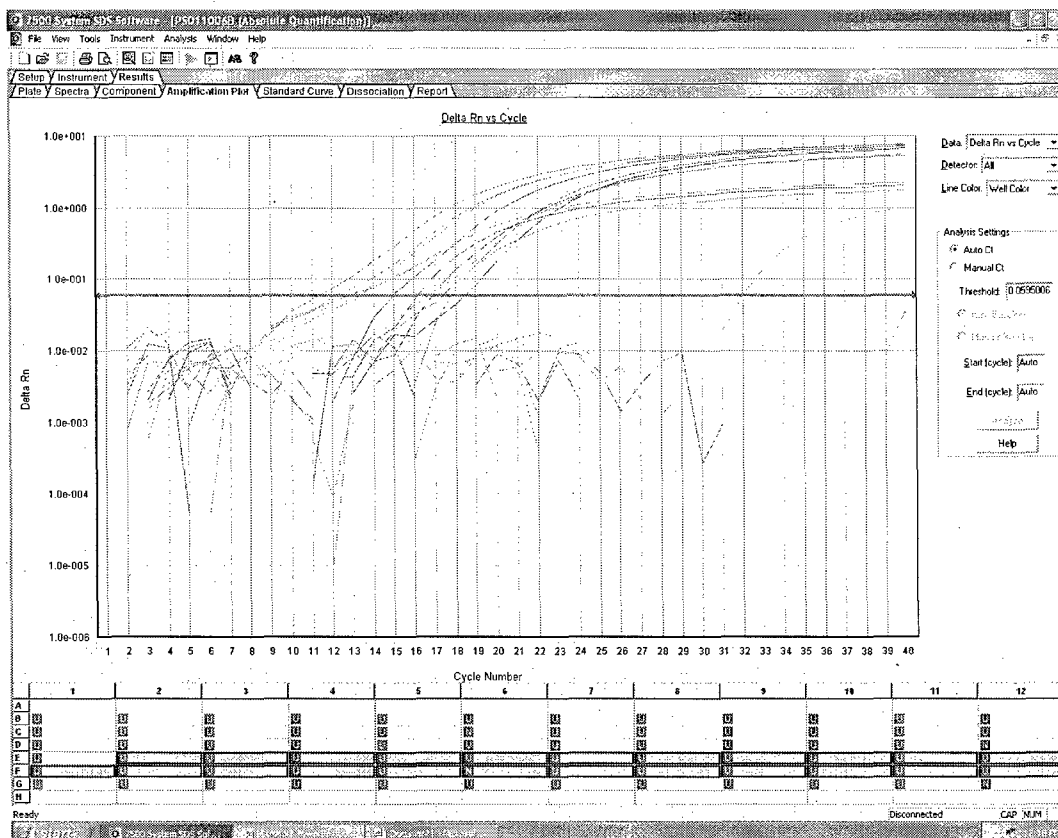


Fig. 2. Example showing the FAM fluorescence detection of *Staphylococcus* spp.

Gram-negative aerobe:

The specificity of the primer/probe set devised for *Pseudomonas aeruginosa* was predicted by comparison to the aligned SSU_rRNA database of the RDP using the CHECK_PROBE utility and was compared to all available 16S rDNA sequences by using the BLAST database search program. Theoretical cross-specificity analysis indicated that the detection system would be specific for *Pseudomonas aeruginosa* at the species level. The primer/probe set was tested against two *Pseudomonas aeruginosa* strains (type strain ATCC 10145 and reference strain ATCC 27853) and the other two *Pseudomonas* strains including the type strain of *Pseudomonas fluorescens* (ATCC 13525^T) and the type strain of *Pseudomonas putida* (ATCC 12633^T). The fluorescent signal was only detected from *Pseudomonas aeruginosa* strains. Specificity of the primer/probe set needs to be further confirmed by testing more phylogenetically related strains in the future.

Table 5. Bacterial strains tested by TaqMan assay

Species	Strains	No.	Staphy- F/R/P	Saur- F/R/P	GAS- F/R/P	GBS- F/R/P	AGS- F/R/P
<i>Staphylococcus</i> spp.							
<i>S. aureus</i>	ATCC 29213	1	+	+	NT	NT	NT
<i>S. aureus</i>	ATCC 25923	1	+	+	NT	NT	NT
<i>S. aureus</i>	Clinical isolates	4	+	+	NT	NT	NT
<i>S. epidermidis</i>	ATCC 12228	1	+	-	NT	NT	NT
<i>S. epidermidis</i>	ATCC 49134	1	+	-	NT	NT	NT
<i>S. epidermidis</i>	Clinical isolates	2	+	-	NT	NT	NT
<i>S. haemolyticus</i>	ATCC 29970 ^T	1	+	-	NT	NT	NT
<i>S. haemolyticus</i>	WAL 1020	1	+	-	NT	NT	NT
<i>S. hominis</i>	WAL 937	1	+	-	NT	NT	NT
<i>S. lugdunensis</i>	ATCC 49576	1	+	-	NT	NT	NT
<i>S. saprophyticus</i>	ATCC 15305 ^T	1	+	-	NT	NT	NT
<i>S. simulans</i>	ATCC 27851	1	+	-	NT	NT	NT
<i>S. warneri</i>	ATCC 49454	1	+	-	NT	NT	NT
<i>S. warneri</i>	WALA 939	1	+	-	NT	NT	NT
<i>S. xylosus</i>	ATCC 29971 ^T	1	+	-	NT	NT	NT
CoNS <i>Staphylococcus</i>	Clinical isolates	7	+	-	NT	NT	NT
<i>Streptococcus</i> spp.							
<i>S. bovis</i>	ATCC 35034	1	-	NT	-	-	-
<i>S. dysgalactiae</i> subsp. <i>equisimilis</i>	ATCC 35666	1	NT	NT	-	-	-
<i>S. mitis</i>	ATCC 49456 ^T	1	-	NT	-	-	-
<i>S. mutans</i>	ATCC 25175 ^T	1	-	NT	-	-	-
<i>S. oralis</i>	ATCC 9811	1	NT	NT	-	-	-
<i>S. salivarius</i>	ATCC 7073 ^T	1	-	NT	-	-	-
<i>S. sanguinis</i>	ATCC 10556 ^T	1	-	NT	-	-	-
<i>S. vestibularis</i>	ATCC 49124 ^T	1	-	NT	-	-	-
<i>S. anginosus</i>	CCUG 27298 ^T	1	NT	NT	NT	-	+
<i>S. anginosus</i>	Clinical isolates	3	NT	NT	NT	NT	+
<i>S. agalactiae</i>	ATCC 12386	1	NT	NT	-	+	-
<i>S. agalactiae</i>	ATCC 13813 ^T	1	NT	NT	-	+	-

<i>S. agalactiae</i>	Clinical isolates	2	NT	NT	NT	+	NT
<i>S. iniae</i>	ATCC 29178 ^T	1	NT	NT	NT	-	-
<i>S. constellatus</i> subsp. <i>constellatus</i>	ATCC 27823 ^T	1	NT	NT	-	-	+
<i>S. constellatus</i>	CCUG 24889	1	NT	NT	NT	-	+
<i>S. constellatus</i>	WALA 154	1	NT	NT	NT	NT	+
<i>S. intermedius</i>	CCUG 32759	1	NT	NT	NT	NT	+
<i>S. intermedius</i>	Clinical isolates	2	NT	NT	NT	NT	+
<i>S. pneumoniae</i>	ATCC 35088	1	NT	NT	-	-	-
<i>S. pneumoniae</i>	ATCC 49615	1	NT	NT	NT	-	NT
<i>S. pneumoniae</i>	ATCC 6305	1	NT	NT	NT	-	NT
<i>S. pyogenes</i>	ATCC 12384	1	NT	NT	+	-	-
<i>S. pyogenes</i>	ATCC 19615	1	NT	NT	+	-	-
<i>S. pyogenes</i>	Clinical isolates	2	NT	NT	+	-	NT

NT: not tested

KEY RESEARCH ACCOMPLISHMENTS

Rapid identification of pathogens involved in wound infections is critical for successfully controlling, managing and eradicating this type of infection. Rapid field-based tests to detect the pathogens involved in the infections are not available. The most significant accomplishment during the last year was development of a rapid real-time TaqMan PCR assay for most of the pathogens encountered in wound infections. This rapid and specific assay provides a new diagnostic tool that conceivably will redefine wound infection management and control strategies for this important problem in both military and civilian life. Evaluation of the sensitivity of the assay is underway.

REPORTABLE OUTCOMES

When the entire set of primers and probes has been completed, this information plus the methodology for real-time PCR using these will be published.

CONCLUSION

We have developed a real-time PCR based high throughput detection method for certain important wound pathogens.

REFERENCES

1. Johnson,J.T. & Yu,V.L. Role of anaerobic bacteria in postoperative wound infections following oncologic surgery of the head and neck. *Ann. Otol. Rhinol. Laryngol.* **100** (Suppl.154), 46-48 (1991).
2. Zoutman,D., McDonald,S. & Vethanayagan,D. Total and attributable costs of surgical-wound infections at a Canadian tertiary-care center. *Infect. Control Hosp. Epidemiol.* **19**, 254-259 (1998).
3. Andrews,J.M. & Wise,R. A survey of susceptibility testing of anaerobes in the United Kingdom. *J.Antimicrob Chemother.* **50**. (2002).
4. Barenfanger,J., Drake,C.A., Lawhorn,J., Kopec,C. & Killiam,R. Outcomes of improved anaerobic techniques in clinical microbiology. *Clin.Infect.Dis.* **35**(Suppl.1), S78-S83. (2002).
5. Bae,HG., Nitsche,A., Teichmann,A., Biel,S.S. & Niedrig,M. Detection of yellow fever virus: a comparison of quantitative real-time PCR and plaque assay. *J.Virol.Methods* **110**, 185-191. (2003).
6. Gu,Z., Belzer,C.S., Gibson,M.J., Bankowski,M.J. & Hayden,R.T. Multiplexed, real-time PCR for quantitative detection of human adenovirus. *J.Clin.Microbiol.* **41**, 4636-4641. (2003).
7. Ikewaki,J. *et al.* Real-time PRC assay compared to nested PCR and antigenemia assays for detecting cytomegalovirus reactivation in adult T-cell leukemia-lymphoma patients. *J.Clin.Microbiol.* **41**, 4382-4387. (2003).
8. Mackay,I.M., Arden,K.E. & Nitshe,A. Real-time PCR in virology. *Nucleic Acid Research* **30**, 1292-1305. (2002).
9. Suzuki,Y. *et al.* Quantitative analysis of human immunodeficiency virus type 1 DNA dynamics by real-time PCR: integration efficiency in stimulated and unstimulated peripheral blood mononuclear cells. *Virus Genes* **27**, 177-188 (2003).
10. Bieche,I. *et al.* Novel approach to quantitative polymerase chain reaction using real-time detection: application to the detection of gene amplification in breast cancer. *Int. J. Cancer* **78**, 661-666 (1998).
11. Dolken,L., Schuler,F. & Dolken,G. Quantitative detection of t(14;18)-positive cells by real-time quantitative PCR using fluorogenic probes. *Biotechniques* **25**, 1058-1064 (1998).

12. Luthra,R., McBride,J.A., Cabanillas,F. & Sarris,A. Novel 5' exonuclease-based real-time PCR assay for the detection of t(14;18)(q32;q21) in patients with follicular lymphoma. *Am. J. Pathol.* **153**, 63-68 (1998).
13. Lascols,C. *et al.* Fast and accurate quantitative detection of *Helicobacter pylori* and identification of clarithromycin resistance mutations in *H. pylori* isolates from gastric biopsy specimens by real-time PCR. *J. Clin. Microbiol.* **41**, 4573-4577 (2003).
14. Maeda,H. *et al.* Quantitative real-time PCR using TaqMan and SYBR Green for *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *tetQ* gene and total bacteria. *FEMS Immunol. Med. Microbiol.* **39**, 81-86 (2003).
15. Rondini,S., Mensah-Quainoo,E., Troll,H., Bodmer,T. & Pluschke,G. Development and application of real-time PCR assay for quantification of *Mycobacterium ulcerans* DNA. *J. Clin. Microbiol.* **41**, 4231-4237 (2003).
16. Saukkoriipi,A., Palmu,A., Kilpi,T. & Leinonen,M. Real-time quantitative PCR for the detection of *Streptococcus pneumoniae* in the middle ear fluid of children with acute otitis media. *Mol. Cell Probes* **16**, 385-390 (2002).
17. Saukkoriipi,A., Palmu,A., Kilpi,T. & Leinonen,M. Real-time quantitative PCR for the detection of *Streptococcus pneumoniae* in the middle ear fluid of children with acute otitis media. *Mol. Cell Probes* **16**, 385-390 (2002).
18. Welti,M. *et al.* Development of a multiplex real-time quantitative PCR assay to detect *Chlamydia pneumoniae*, *Legionella pneumophila* and *Mycoplasma pneumoniae* in respiratory tract secretions. *Diagn. Microbiol. Infect. Dis.* **45**, 85-95 (2003).
19. Yoshida,A. *et al.* Development of a 5' fluorogenic nuclease-based real-time PCR assay for quantitative detection of *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis*. *J.Clin.Microbiol.* **41**, 863-866. (2003).
20. Higgins,D. *et al.* CLUSTAL W: improving the sensitivity of progressive multiple sequence alingment through sequence weighting, position-specific gap penalties and weigh matrix choice. *Nucleic Acid Research* **22**, 4673-4680. 1994.
21. Maidak,B.L. *et al.* The RDP-II (Ribosomal Database Project). *Nucleic Acid Research* **29**, 173-174. (2001).
22. benson,D.A. *et al.* GenBank. *Nucleic Acid Research* **30**, 17-20. 2002.
23. Liu D, Hollingshead S, Swiatlo E, Lawrence ML, Austin FW. Rapid identification of *Streptococcus pyogenes* with PCR primers from a putative transcriptional regulator gene. *Res Microbiol.* 156(4): 564-7. 2005

24. Bergh K, Stoelhaug A, Loeseth K, Bevanger L. Detection of group B streptococci (GBS) in vaginal swabs using real-time PCR with TaqMan probe hybridization. *Indian J Med Res.* **119** Suppl:221-3. 2004
25. Takao A, Nagamune H, Maeda N. Identification of the anginosus group within the genus *Streptococcus* using polymerase chain reaction. *FEMS Microbiol Lett.* **233**: 83-9. (2004)
26. Sakai H, Procop GW, Kobayashi N, Togawa D, Wilson DA, Borden L, Krebs V, Bauer TW. Simultaneous detection of *Staphylococcus aureus* and coagulase-negative staphylococci in positive blood cultures by real-time PCR with two fluorescence resonance energy transfer probe sets. *J Clin Microbiol.* **42**:5739-44. 2004
27. Hein I, Lehner A, Rieck P, Klein K, Brandl E, Wagner M. Comparison of different approaches to quantify *Staphylococcus aureus* cells by real-time quantitative PCR and application of this technique for examination of cheese. *Appl Environ Microbiol.* **67**, 3122-6. (2001)
28. Martin FE, Nadkarni MA, Jacques NA, Hunter N. Quantitative microbiological study of human carious dentine by culture and real-time PCR: association of anaerobes with histopathological changes in chronic pulpitis. *J Clin Microbiol.* **40**, 1698-704. (2000)
29. Wise MG, Siragusa GR. Quantitative detection of *Clostridium perfringens* in the broiler fowl gastrointestinal tract by real-time PCR. *Appl Environ Microbiol.* **71**, 3911-6 (2005)
30. Vandecasteele SJ, Peetermans WE, Merckx R, Van Eldere J. Quantification of expression of *Staphylococcus epidermidis* housekeeping genes with Taqman quantitative PCR during in vitro growth and under different conditions. *J Bacteriol.* **183**, 7094-101. (2001)
31. McDonald RR, Antonishyn NA, Hansen T, Snook LA, Nagle E, Mulvey MR, Levett PN, Horsman GB. Development of a triplex real-time PCR assay for detection of Panton-Valentine leukocidin toxin genes in clinical isolates of methicillin-resistant *Staphylococcus aureus*. *J Clin Microbiol.* **43**, 6147-9. 2005